myT® KRAS

qPCR primers for detection of seven human KRAS codon 12/13 mutations:

KRAS G12D: Gly12Asp (GGT→GAT)

KRAS G12A: Gly12Ala (GGT→GCT)

KRAS G12V: Gly12Val (GGT→GTT)

KRAS G12S: Gly12Ser (GGT→AGT)

KRAS G12R: Gly12Arg (GGT→CGT)

KRAS G12C: Gly12Cys (GGT→TGT)

KRAS G13D: Gly13Asp (GGC→GAC)

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Prime Time® qPCR Probes is a registered trademark and product of Integrated DNA Technologies

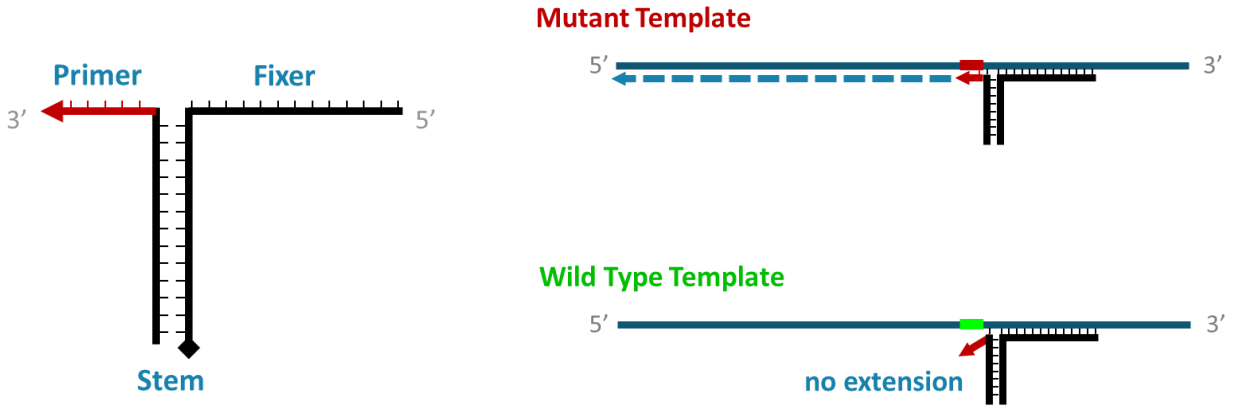
QIAamp® is a registered trademark and product of Qiagen GmbH

ABI™ 7500 Real Time PCR System is a trademark and product of Applied Biosystems, now part of Life Technologies Corp.

myT® Primer Technology

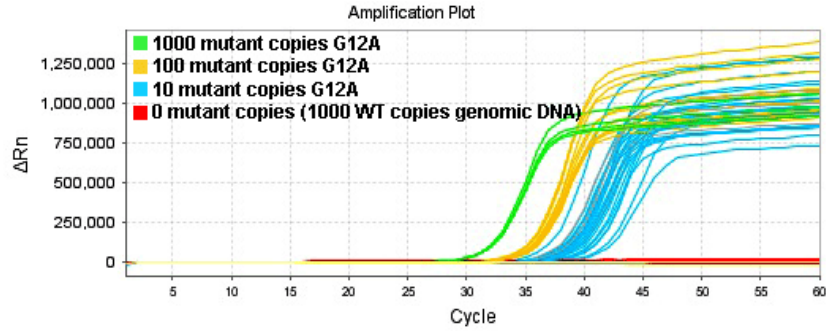
myT Primers have unique structural and thermodynamic properties that make them highly sensitive to mismatch discrimination. myT Primers are comprised of Primer and Fixer oligonucleotides with three functional domains: the long Fixer domain provides a high level of specificity for genomic DNA templates, the Primer domain is highly sensitive to single base mutations due to its very short length, and the double stranded stem links the Fixer and Primer domains.

When a mutant-specific myT Primer is combined with a reverse primer and hydrolysis probe, myT Primers can detect 1% mutant KRAS codon 12/13 mutations present in a background of 10^3 wild-type genomic DNA copies without non-specific amplification from wild-type; either a positive or negative amplification signal is generated and a delta Ct method to distinguish specific from non-specific amplification is not required (see data on page 4).

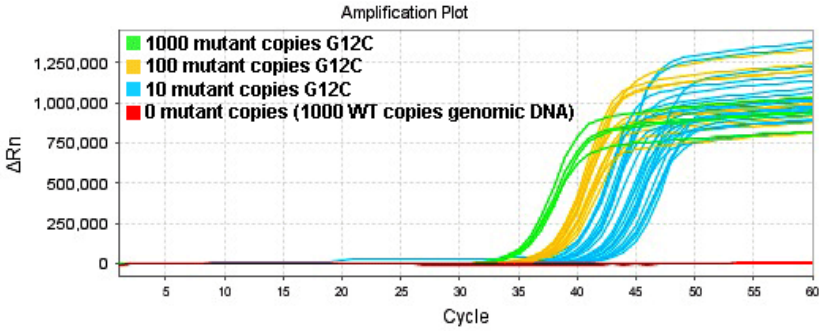


myT KRAS Performance

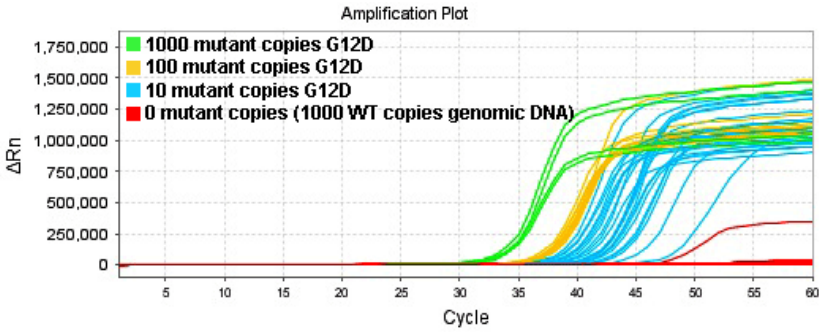
G12A



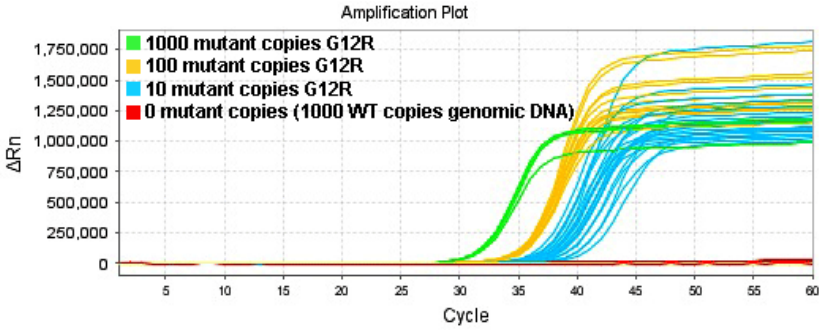
G12C



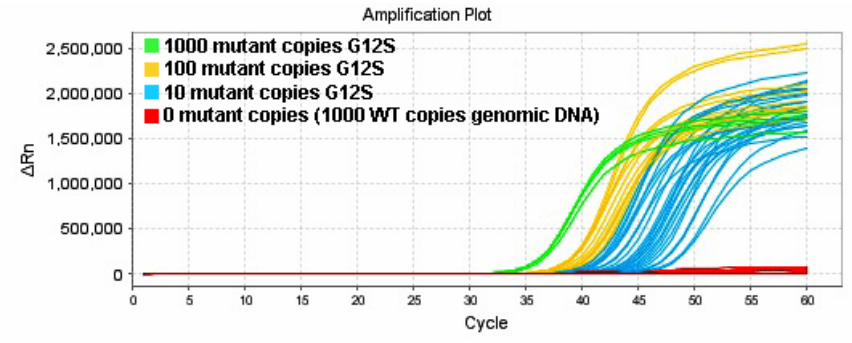
G12D



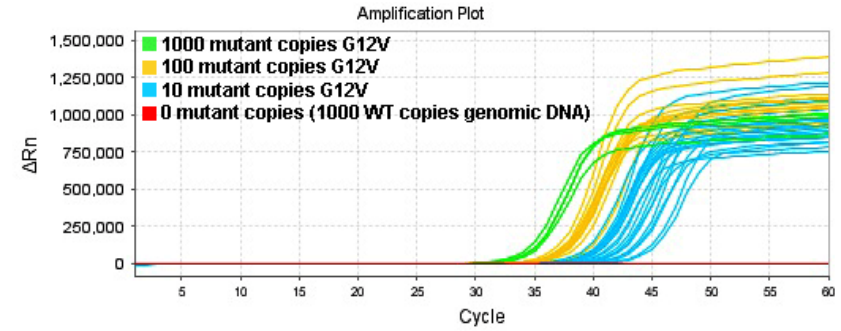
G12R



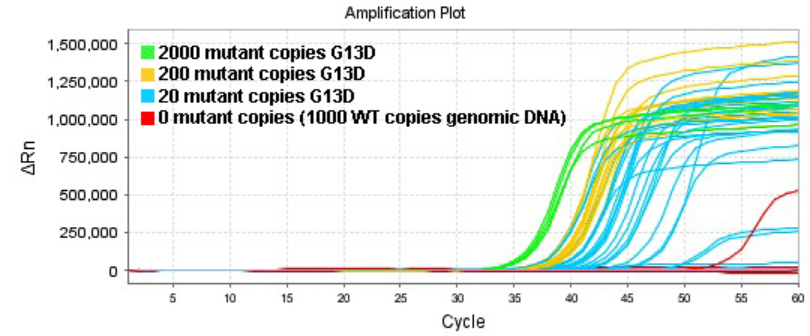
G12S



G12V



G13D



Amplification plots. qPCR reactions containing mutant genomic DNA at the specified quantity in a background of 10^3 wild-type genomic DNA (red; n = 16 replicates per assay) resulted in KRAS mutant-specific amplification: 50% mutant content (green; n = 4 replicates per assay), 10% mutant content (yellow; n = 12 replicates per assay) and 1% mutant content (blue; n = 24 replicates per assay). Assays performed on an ABI 7500.

Conclusion. These results demonstrate mismatch discrimination with very high specificity. The results are clear and unambiguous, eliminating the need for ΔCt analysis to distinguish specific from non-specific amplification. This high confidence, "Yes/No" clarity is a feature that is exclusive to myT Primer reagents.

Protocol

The myT KRAS kit provides sufficient reagents to perform a total of 30 assays to assess KRAS codon 12/13 mutations using the ABI 7500 Real Time PCR System.

Mutation detection with myT KRAS consists of two steps:

1. Locus-specific qPCR
   - A non-allele-specific qPCR is performed to assess total (mutant + wild-type) amplifiable KRAS for each sample
   - This determines the quantity of DNA to be used in the allele-specific PCR for each sample
   - Reagents for 30 reactions, including controls, are included
   - One locus-specific PCR reaction per DNA sample is performed

2. Allele-specific qPCR
   - A mutant allele-specific KRAS qPCR is then performed to assess presence of codon 12/13 mutations
   - Results are reported as positive or negative for mutant KRAS for each sample with a sensitivity limit of 1% (10 mutant copies in 10^3 wild-type copies)
   - Reagents for 30 reactions per allele, including controls, are included
   - For each DNA sample, seven separate allele-specific PCR reactions are performed

Reagents Included

Reagent Mixes	Volume	Description
KRAS Locus-specific Primers	198 µl	Non-allele-specific myT KRAS primers
KRAS G12D Primers	198 µl	Allele-specific myT KRAS primers
KRAS G12A Primers	198 µl	Allele-specific myT KRAS primers
KRAS G12V Primers	198 µl	Allele-specific myT KRAS primers
KRAS G12S Primers	198 µl	Allele-specific myT KRAS primers
KRAS G12R Primers	198 µl	Allele-specific myT KRAS primers
KRAS G12C Primers	198 µl	Allele-specific myT KRAS primers
KRAS G13D Primers	198 µl	Allele-specific myT KRAS primers
Nuclease-free Buffer	1 ml	For DNA sample dilution and NTC* reactions

***NTC = no-template control**

Shipped in a separate box:

KRAS mixed DNA Standard	180 µl	KRAS mixed mutant DNA (200 copies/µl total)

- Store all reagents at -20° C upon arrival
- To avoid cross-contamination, store the myT Primers box separately from the DNA Standard box
- Refreeze unused myT Primers and DNA Standard at -20° C
- For best performance, limit freeze-thaw cycles to 4

Reagents not included

Reagents	Recommended Vendor
EagleTaq Master Mix	Roche part number 05529085 190
Dual-Labeled Probe	IDT PrimeTime Dual-Labeled Probe Free of charge – voucher provided

Note: *myT KRAS has been optimized for use with the above reagents. Reagents from other vendors may be substituted, but substitutions may result in reduced assay performance or require the user to modify assay conditions to achieve maximal performance.*

Probe sequence

5'- /56-FAM/AGCTGTATCGTCAAGGCACTCTTGCC/3IABkFQ/ -3'

- When ordering this probe, please include an internal Zen quencher
- Details on how to redeem the free of charge voucher for the dual-labeled probe from IDT were sent with your order acknowledgement. If you have any questions, please contact Swift Technical Support at 734.330.2568 or technicalsupport@swiftbiosci.com.

Instructions for re-suspension of probe

- Spin lyophilized probe to collect contents
- Resuspend in Nuclease-free Buffer provided to achieve a 100 μM stock based on actual yield obtained
- Make a 3 μM working dilution of the probe
- Distribute 72 μl each to the Locus-specific and seven Allele-specific myT Primer stocks
- The final volume for each stock will be 198 + 72 = 270 μl
- Probes are light-sensitive. Avoid prolonged exposure to light once probe has been added.

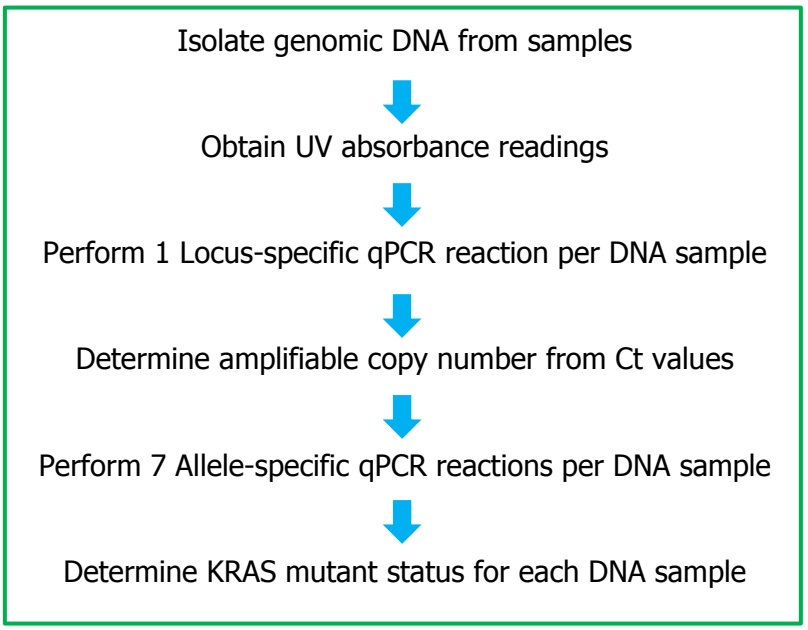
96-well plates are not supplied, but the following have been tested using myT KRAS:

- ABI 7500: Order from Applied Biosystems/Life Technologies
  - 96-well optical reaction plates cat. no. 4306737
  - MicroAmp optical Adhesive Film cat. no. 4311971

Notes Regarding DNA Samples

- For high quality DNA derived from cell lines or fresh-frozen clinical samples, UV absorbance readings correlate well with amplifiable content.
- For DNA derived from formalin-fixed paraffin embedded samples (FFPE), UV absorbance readings determine the DNA concentration but do NOT accurately determine amplifiable content due to DNA damage from fixation.
- It is recommended to obtain UV absorbance readings for each sample in order to determine the amount of DNA to use in the Locus-specific qPCR (Step 1).
- It is recommended to use ~5 ng of high quality DNA or a range of 10 – 50 ng of FFPE DNA for the Locus-specific qPCR.
- In the case of heavily damaged samples, >50 ng DNA can be placed into a reaction, but inhibition of PCR may occur. Similarly, it is not recommended to place greater than 20% volume of DNA per 25 µl reaction as PCR inhibitors are present in some FFPE samples.
- This assay has been tested using DNA isolated by the Qiagen QIAamp DNA FFPE Tissue Kit with RNase treatment (not included in this kit). Since RNA co-purifies with DNA, RNase treatment provides more accurate DNA quantification based on UV absorbance reading.
- To avoid cross-contamination that could lead to false positive results:
    - Change gloves frequently
    - Use aerosol-resistant pipette tips
    - Use pipettes dedicated for template and non-template containing reagents
    - Maintain separate work areas for template and non-template containing reagents
    - Routinely decontaminate work areas with 10% bleach and/or UV light
    - Never open PCR reaction wells that resulted in allele-specific amplification

myT KRAS Workflow


Isolate genomic DNA from samples
Obtain UV absorbance readings
Perform 1 Locus-specific qPCR reaction per DNA sample
Determine amplifiable copy number from Ct values
Perform 7 Allele-specific qPCR reactions per DNA sample
Determine KRAS mutant status for each DNA sample

The contents provided are sufficient to perform 240 reactions consisting of 30 Locus-specific and 30 Allele-specific reactions per allele for seven different mutations (8 X 30). This enables testing of up to 28 samples when including a positive control and NTC if performed as a single qPCR run. If testing is split into multiple batches, total samples tested will be less since a positive control and NTC are required for each run. For example, if testing is batched into 3 qPCR runs, the total number of samples analyzed will be 24 (8 per run) where 3 positive control and 3 NTC reactions are also run. Positive control template (mixed DNA Standard) is provided in sufficient quantity for up to 4 separate batch runs per kit. For all included reagents, a 10% excess volume is included to compensate for pipetting loss.

Step 1: Locus-specific KRAS qPCR

Thaw reagents completely at room temperature. Once thawed, invert repeatedly or gently vortex and briefly centrifuge to collect contents. To avoid cross-contamination, always briefly centrifuge DNA Standard and DNA samples prior to opening caps. Also, gently mix reactions containing EagleTaq Master Mix to avoid formation of bubbles that can interfere with fluorescence detection.

Each reaction contains:

KRAS Locus-specific Primers + Probe*	7.5 µl
EagleTaq Master Mix	12.5 µl
DNA Template	5 µl
Total Volume	**25 µl**

*Remember to add 72 µl of resuspended 3µM probe to the myT Primer tube before initial use

1. Make a cocktail with KRAS Locus-specific primers and EagleTaq Master Mix in the amount needed for the number of reactions to be run plus up to 5% extra volume to compensate for pipetting loss (maximum = 28 samples plus 2 control wells).
2. Invert tube with the cocktail repeatedly to mix reagents and briefly centrifuge to collect contents.
3. Dispense 20 µl cocktail into each reaction well.
4. Add 5 µl sample DNA corresponding to 5 ng high quality DNA or 10 to 50 ng of FFPE DNA. If necessary, use Nuclease-free Buffer (provided) to dilute samples.
5. Include a "no template control" (NTC) by adding 5 µl Nuclease-free Buffer to one reaction well.
6. Include a 10^3 copy positive control by adding 5 µl KRAS DNA Standard to one reaction well.
7. Seal plate and briefly centrifuge at 1000-2000 RPM for 15 seconds to collect contents.
8. Load plate into the selected thermocycler and follow run instructions (for details, see Appendix for ABI 7500 instructions).

Cycling Temperature	Cycling Time	Cycles
95° C	10 minutes	1 cycle
95° C	14 seconds	**45 cycles**
65° C	1 minute*	

*with FAM read; disable any reads for passive reference dyes such as ROX

Determination of amplifiable copy number for the allele-specific assay

1. The control DNA Standard has 10^3 amplifiable KRAS copies per 5 µl and should have a Ct value as specified in the table below if using the ABI 7500. 10^3 is the recommended amplifiable copy number to place in the Allele-specific assay. Limiting the assay to 10^3 amplifiable copies reduces the likelihood of PCR inhibition and detection of low-level cross contamination that can be present in FFPE samples.

Thermocycler	Expected Average Ct value for Locus-specific 10^3 copies
ABI 7500	28.4

2. If samples have a Ct value less than the control DNA well, dilute with Nuclease-free Buffer to 10^3 amplifiable copies per 5 µl, assuming that a 2-fold dilution will increase the Ct value by 1.

   Example: If a Ct of 27.4 is obtained, 28.4-27.4 = 1 Ct, so dilute sample 2-fold

3. If samples have a Ct value greater than the control DNA well, add up to 10^3 amplifiable copies per 5 µl, assuming that a two-fold increase in DNA will decrease the Ct value by 1. Do not exceed 20% DNA per reaction volume, as PCR inhibitors are present in FFPE preparations.

   Example: If a Ct of 30.4 is obtained, 30.4-28.4= 2, so add 4-fold more DNA, if possible

4. If samples have insufficient amplifiable copy number, 1% sensitivity is not likely to be achieved as 1% represents 10 mutant copies in 10^3 total copies. Based on Poisson distribution, copy number less than 10 is not detected at 100% frequency in a single well reaction.

5. If the Locus-specific Ct value is >35, the amplifiable copy number is too low to proceed.

6. Regarding the NTC, either no amplification or an occasional Ct $\geq$38 may be obtained. If the NTC or DNA Standard (positive control) fails, contact technical service.

Step 2: Allele-specific KRAS qPCR

Thaw reagents completely at room temperature. Once thawed, invert repeatedly or gently vortex and briefly centrifuge to collect contents. To avoid cross-contamination, always briefly centrifuge DNA Standard and DNA samples prior to opening caps. Also, gently mix reactions containing EagleTaq Master Mix to avoid formation of bubbles that can interfere with fluorescence detection.

Each of the seven allele-specific reactions contains:

KRAS Allele-specific Primers (G12D, G12A, G12V, G12S, G12R, G12C, or G13D) + Probe*	7.5 µl
EagleTaq Master Mix	12.5 µl
DNA Template	5 µl
Total Volume	**25 µl**

*Remember to add 72 µl of resuspended 3 µM probe to the myT Primer tubes before initial use

1. Make seven cocktails with each KRAS Allele-specific Primer reagent and EagleTaq Master Mix in the amount needed for the number of samples to be run plus up to 5% extra volume to compensate for pipetting loss (maximum = 28 samples plus 2 control wells).
2. Invert the cocktails repeatedly to mix reagents and briefly centrifuge to collect contents.
3. Dispense 20 µl cocktail into each reaction well. [Suggested 96-well plate layout: place the seven allele-specific assays in rows and up to 10 samples plus controls in columns].
4. For each sample, add 5 µl DNA that corresponds to 10^3 amplifiable copies into each of the 7 allele-specific reaction wells (DNA amount determined from the Locus-specific qPCR above).
5. For each allele-specific cocktail include a "no template control" (NTC) by adding 5 µl Nuclease-free Buffer to one reaction well.
6. For each allele-specific cocktail include a 10^3 copy positive control by adding 5 µl mixed DNA Standard to one reaction well.
7. Seal plate and briefly centrifuge 1000-2000 RPM for 15 seconds to collect contents.
8. Load plate into the selected thermocycler and follow run instructions (for details, see Appendix for ABI 7500 instructions).

Cycling Temperature	Cycling Time	Cycles
95° C	10 minutes	1 cycle
95° C	14 seconds	**60 cycles**
65° C	1 minute*	

*with FAM read; disable any reads for passive reference dyes such as ROX

Determination of KRAS mutation status for each sample

- For each Allele-specific myT KRAS qPCR, either a positive or negative amplification signal will be obtained.
- If 10^3 amplifiable copies are analyzed, a 1% sensitivity limit which represents 10 mutant copies in 10^3 wild-type copies can be achieved.
- If only 10^2 amplifiable copies are analyzed, a reduced 10% sensitivity limit can be achieved which represents 10 mutant copies in 10^2 wild-type copies.
- The cut-off Ct values for detection of 10 mutant copies for the thermocycler tested are in the table below.

Thermocycler	**10 Copy Ct Cut-off**						
	G12D	G12V	G12A	G12R	G12C	G12S	G13D
ABI 7500	45	44	44	40	45	47	47*

*20 copy Ct cut-off

- If a Ct value is obtained that exceeds the cut-off, it is scored as negative or below the limit of detection for this assay.
- Occasionally when amplifiable copy number is limiting, a Ct value near the cut-off will be obtained. In this case, the assay can be repeated to confirm a positive amplification signal.
- If positive, the Allele-specific Ct value will be dependent on the percent tumor cell content and the tumor heterogeneity of the sample from which the DNA was derived. Low percentage tumor cell samples will have limited sensitivity.

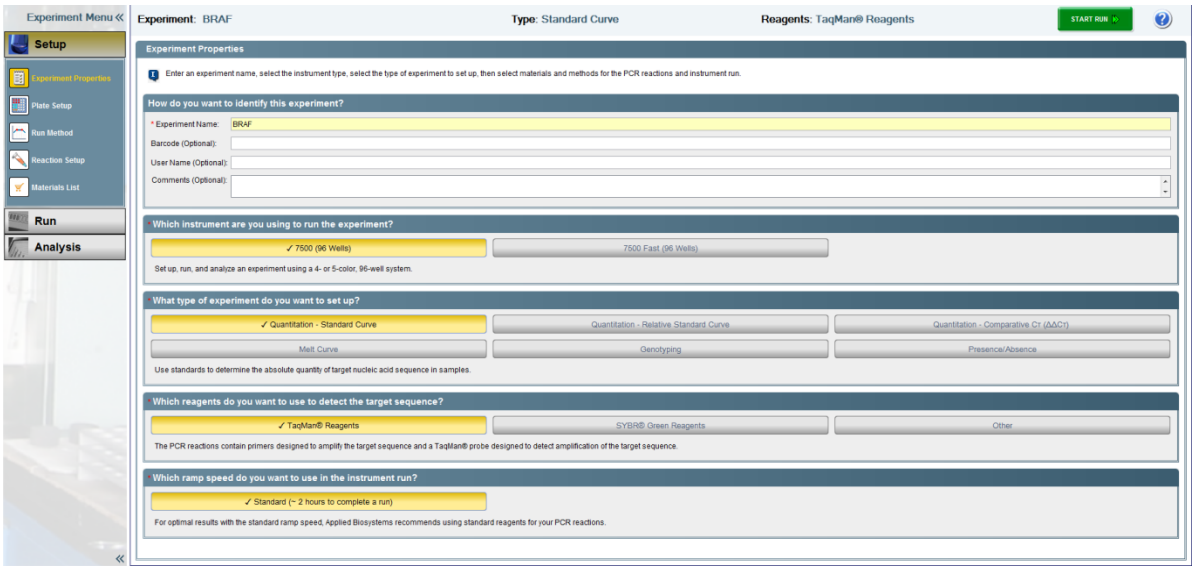
Appendix

Life Technologies ABI 7500 - Run protocol

1. Turn on the ABI 7500
2. Open ABI7500 software on your computer
3. Select "Advanced Setup"

Setup – Experiment properties

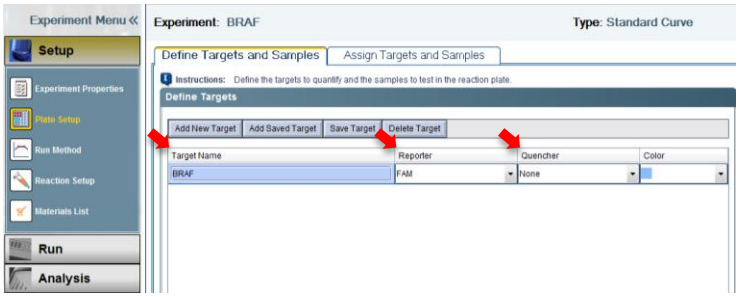
1. Name your experiment
2. Select:
   "7500 (96 Wells)"
   "Quantitation – Standard Curve"
   "TaqMan® Reagents"
   "Standard (~ 2 hours to complete a run)"


Experiment Menu
Experiment: BRAF
Type: Standard Curve
Reagents: TaqMan® Reagents
START RUN
Setup
Experiment Properties
Plate Setup
Run Method
Reaction Setup
Materials List
Run
Analysis
Experiment Properties
Enter an experiment name, select the instrument type, select the type of experiment to set up, then select materials and methods for the PCR reactions and instrument run.
How do you want to identify this experiment?
Experiment Name: BRAF
Barcode (Optional):
User Name (Optional):
Comments (Optional):
Which instrument are you using to run the experiment?
✓ 7500 (96 Wells)
7500 Fast (96 Wells)
Set up, run, and analyze an experiment using a 4- or 5-color, 96-well system.
What type of experiment do you want to set up?
✓ Quantitation - Standard Curve
Quantitation - Relative Standard Curve
Quantitation - Comparative CT (ΔΔCT)
Melt Curve
Genotyping
Presence/Absence
Use standards to determine the absolute quantity of target nucleic acid sequence in samples.
Which reagents do you want to use to detect the target sequence?
✓ TaqMan® Reagents
SYBR® Green Reagents
Other
The PCR reactions contain primers designed to amplify the target sequence and a TaqMan® probe designed to detect amplification of the target sequence.
Which ramp speed do you want to use in the instrument run?
✓ Standard (~ 2 hours to complete a run)
For optimal results with the standard ramp speed, Applied Biosystems recommends using standard reagents for your PCR reactions.

Setup – Plate Setup

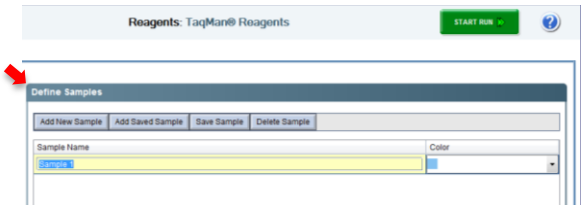
1. Define Targets and Samples (*Define Targets*)

Target Name	Reporter	Quencher	Color
KRAS	FAM	None	your choice



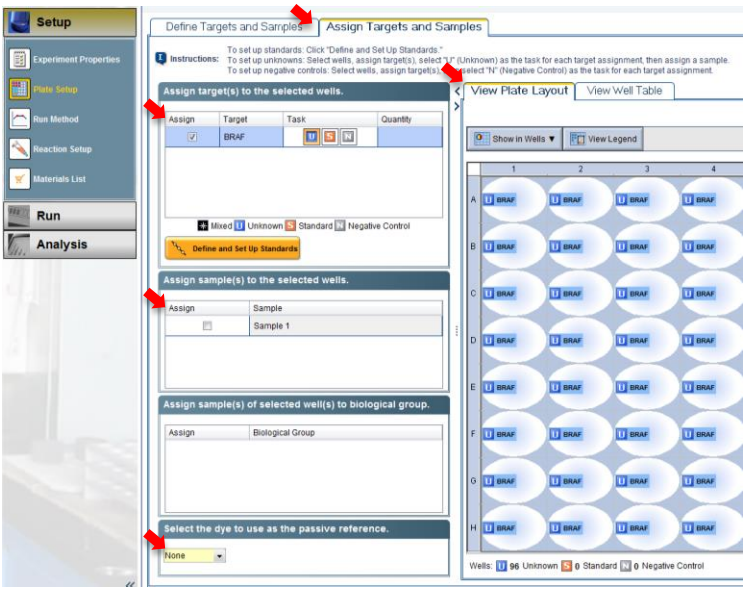
2. Define Targets and Samples (*Define Samples)*

   Name your samples



3. Assign Targets and Samples

- Select each well containing a reaction in "View Plate Layout" and assign "Target KRAS"
- Assign your particular samples the same way
- Select the dye to use as a passive reference "None"

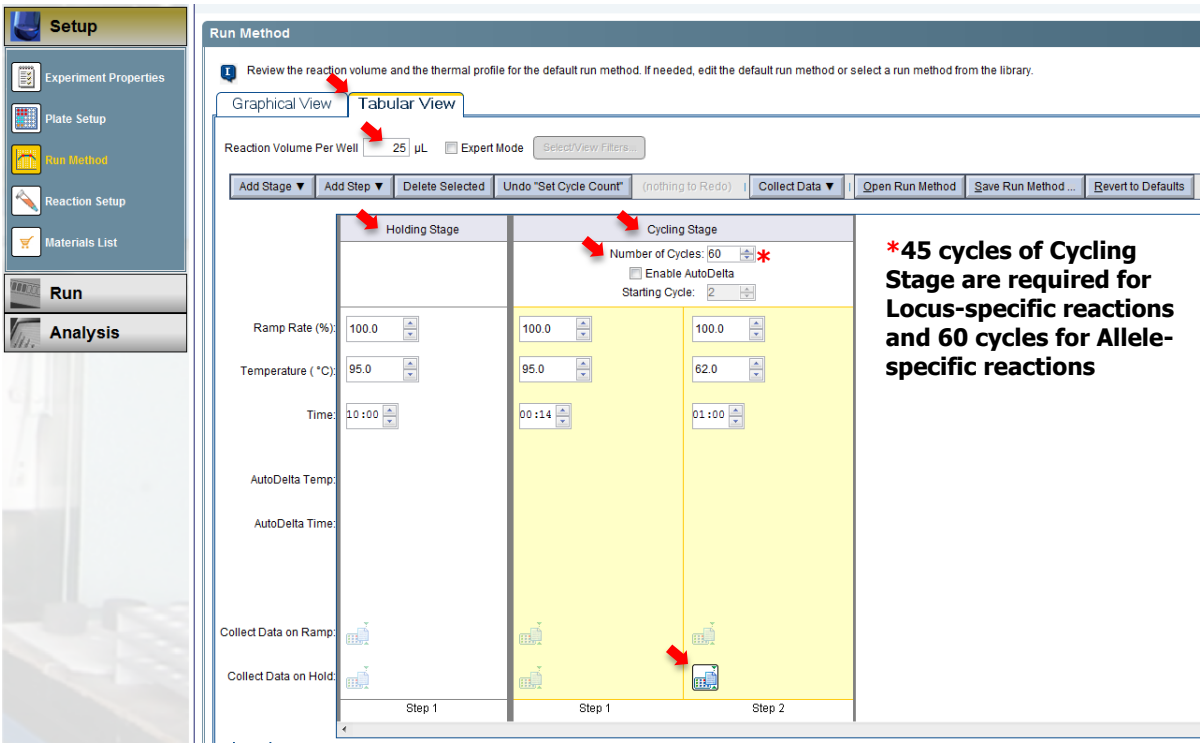


Setup - Run method

1. Select Tabular View
2. Reaction Volume Per Well "25µl"
3. Holding Stage (1 step):
   "95°C, 10 minutes, ramp rate 100%"
4. Cycling Stage (2 steps):
5. Number of Cycles: 45 or 60 cycles*

   "95°C, 14 seconds, ramp rate 100%"
   "65°C, 1 minute, ramp rate 100%" + "collect data on hold"

***45 cycles of Cycling Stage are required for Locus-specific reactions and 60 cycles for Allele-specific reactions**



6. Open the door on the ABI 7500. Insert your plate. Close the door
7. Click on "START RUN" in the upper right corner of the screen